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Joseph E. Zahner
Reg. No. 48,224

In re Application of: Zahner et al.

Serial No.: 09/919,298

Filed: July 31, 2001

For: In vitro derived
pluripotent stem cells
and uses therefor

Examiner: Woitach, Joseph

Group Art Unit: 1632

DECLARATION OF DR. JOSEPH ZAHNER UNDER 37 CFR § 1.132

I, Joseph Edward Zahner, declare and state as follows:

1. I am a co-inventor of U.S. Patent Application No. 09/919,298.

2. I am presently President and Chief Science Officer of the small biotech start-up company named Nucleus Remodeling, Inc., which is the assignee of U.S. Patent Application No. 09/919,298.

3. I am also Patent Agent and Intellectual Property Manager in the Technology Transfer Office of Saint Louis University.

4. I am also adjunct Assistant Professor of Biology at Saint Louis University, where I collaborate with faculty on various cell, developmental and molecular biology projects, as well as teach in the graduate program in that department.

5. I received a Ph.D. in Developmental Biology in 1993 from The Johns Hopkins University in Baltimore.

6. I have reviewed the final office action of June 17, 2003 and have spoken with the Examiner in a telephonic interview regarding alleged particular problems with the application. Briefly, an important issue of contention is whether a pluripotent cell, as reasonably understood by the skilled cell or developmental biologist, has actually been made according to the method described and claimed in the instant patent application.

7. In this declaration, I describe the results of various experiments that demonstrate that the reprogrammed cells, which were created by the method claimed in the instant application, (a) express various markers associated with pluripotent stem cells, and (b) express markers associated with committed, differentiating or differentiated cells upon additional treatment with retinoic

acid, which were not expressed in non-reprogrammed keratinocytes treated with retinoic acid.

8. To demonstrate that the reprogrammed keratinocytes, which were reprogrammed by treating with 5-aza-2′ deoxycytidine, trichostatin A and Tat-cyclin B, were like pluripotent stem cells, the following protein expression experiments were carried out: Non-reprogrammed human keratinocytes ("Non-reprogrammed cells") and reprogrammed keratinocytes, which were treated with 5-aza-2′ deoxycytidine, trichostatin A and Tat-cyclin B ("Reprogrammed cells) were treated with 1 uM retinoic acid for 5 days. Cells were then fixed in 4% formaldehyde in phosphate buffered saline for 10 minutes, followed by 0.5% Triton-X100 for 30 seconds. Cells were washed in PBS then re-suspended in dilute horse serum (VectaStain® Kit, Vector Labs). Primary antibodies were added to a final concentration of 1:1000 and incubated for either 1 hour at 37°C or overnight at 4°C (mouse anti-neurofilament [neuron-specific; "NF-L"] , anti-α-fetoprotein [liver-specific; "AFP"], anti-insulin [beta cell-specific; "Insulin"] and anti-cardiac actin [muscle-specific; "mAct"] were obtained from Santa Cruz Biotechnology; anti-stage specific embryonic antigen-1 [embryo-specific; "SSEA-1"] was obtained from the University of Iowa Hybridoma Center). Antibody detection was obtained using the VectaStain Kit (Vector Labs) following manufacturer's instructions.

To determine the proportion of cells that express a given protein, stained versus unstained cells were counted and the proportion of stained cells in the appropriate "no primary" control was subtracted from the proportion of stained cells for each primary antibody. The results of these experiments are presented in Figure 1 of EXHIBIT 1. Each bar represents at least two experiments. Total number of cells for each experiment (n) >300.

9. The results of the foregoing experiments demonstrate that 19.9% ± 5.7% of the reprogrammed keratinocytes (prior to retinoic acid treatment) express SSEA-1 protein, whereas 26.4% ± 2.0% of the reprogrammed keratinocytes (subsequent to retinoic acid treatment) express SSEA-1 protein. This result exactly mirrors those observations made by Henderson et al., *Stem Cells* 20:329-337, 2002, which teaches that human embryonic stem cells increase their expression of SSEA-1 *after* retinoic acid treatment (see Figure 1, panel B on page 331 of that paper.) Cell and developmental biologists reasonably expect that (in the case of human cells) only differentiating embryonic stem cells express SSEA-1 protein. Thus, since the number of reprogrammed human keratinocytes, which have undergone the 5-aza-2′ deoxycytidine, trichostatin A, Tat-cyclin B treatment regimen as described in the subject patent application, express SSEA-1 in practically the same proportion as human embryonic stem cell lines hES H7 and hES H14 (see again Henderson, figure 1), the cell and developmental biologist would reasonably expect the reprogrammed keratinocytes to be human embryonic stem cell-like.

In the controls, 0.00% ± 0.00% of the non-reprogrammed keratinocytes (prior to retinoic acid treatment) express SSEA-1 protein, whereas 6.1% ± 2.3% of the non-reprogrammed keratinocytes (subsequent to retinoic acid treatment) express SSEA-1 protein.

10. The results of the foregoing experiment also clearly demonstrate that a significant proportion of the reprogrammed cells, after retinoic acid treatment, express proteins that are associated with cell-types representative of tissues derived from any of the three primordial germ layers (*i.e.*, 15.4% ± 6.5% for neurofilament protein production, 21.4% ± 7.1% for muscle-specific actin, 24.4% ±

4.0% for insulin, and 28.4% ± 1.5% for α-fetoprotein production). These proteins are not expressed (or expressed in very few cells) by the control keratinocytes treated similarly with retinoic acid. Given that such a diverse set of proteins are produced by the reprogrammed cell treated with retinoic acid, the skilled cell and developmental biologist would reasonably expect that the reprogrammed keratinocytes are pluripotent.

11. As described in the specification of the subject patent application, expression of the transcription factor Oct-4 is associated embryonal carcinoma cells, embryonic stem cells and embryonic germ cells. Furthermore, recent evidence suggests that Oct-4 is required for the pluripotency of embryonic stem cells (see Buehr et al., *Biology of Reproduction* 68:222-229, 2003.)

I have shown by RT-PCR using human Oct-4 specific primers, that keratinocytes reprogrammed by treatment with 5-aza-2′ deoxycytidine, trichostatin A and tat-cyclin B, express Oct-4, whereas non-reprogrammed keratinocytes do not. Figure 2 of EXHIBIT 2 shows an ethidium bromide stained gel of the RT-PCR experiment after 40 cycles of PCR. The positive control lane included a template cDNA of human embryonal carcinoma Oct 4 obtained through the IMAGE consortium.

12. I have demonstrated that keratinocytes, which have been reprogrammed by treating with 5-aza-2′ deoxycytidine, express Oct4 mRNA, telomerase mRNA ans SSEA-1 protein. The expression patterns of those molecular markers have been found, heretofore, only to be associated with embryonic stem cells or the like.

Furthermore, the expression of non-keratinocyte mRNAs and proteins (mRNAs: neurofilament heavy chain, α1-antitrypsin, and cardiac actin; proteins:

insulin, neurofilament light chain, α fetoprotein, and muscle-specific actin; all of which represent cell-types derived from all three primordial germ layers) by reprogrammed keratinocytes, which were subsequently treated with retinoic acid to affect differentiation, demonstrates that the reprogrammed keratinocytes do in fact have a very wide potential to express genes of a multitude of lineages. In other words, the reprogrammed keratinocytes show pluripotency.

Therefore, it is reasonable to conclude that the reprogrammed keratinocytes of the instant invention are very similar to pluripotent stem cells. These cells, given their proven plasticity, may be valuable as a research tool for understanding complex developmental gene regulation processes or for developing protocols for forced cell differentiation.

13. I further declare that all statements herein made by my own knowledge are true and all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the above-identified application.

Dated: August 29, 2003

Joseph Edward Zahner, Ph.D.

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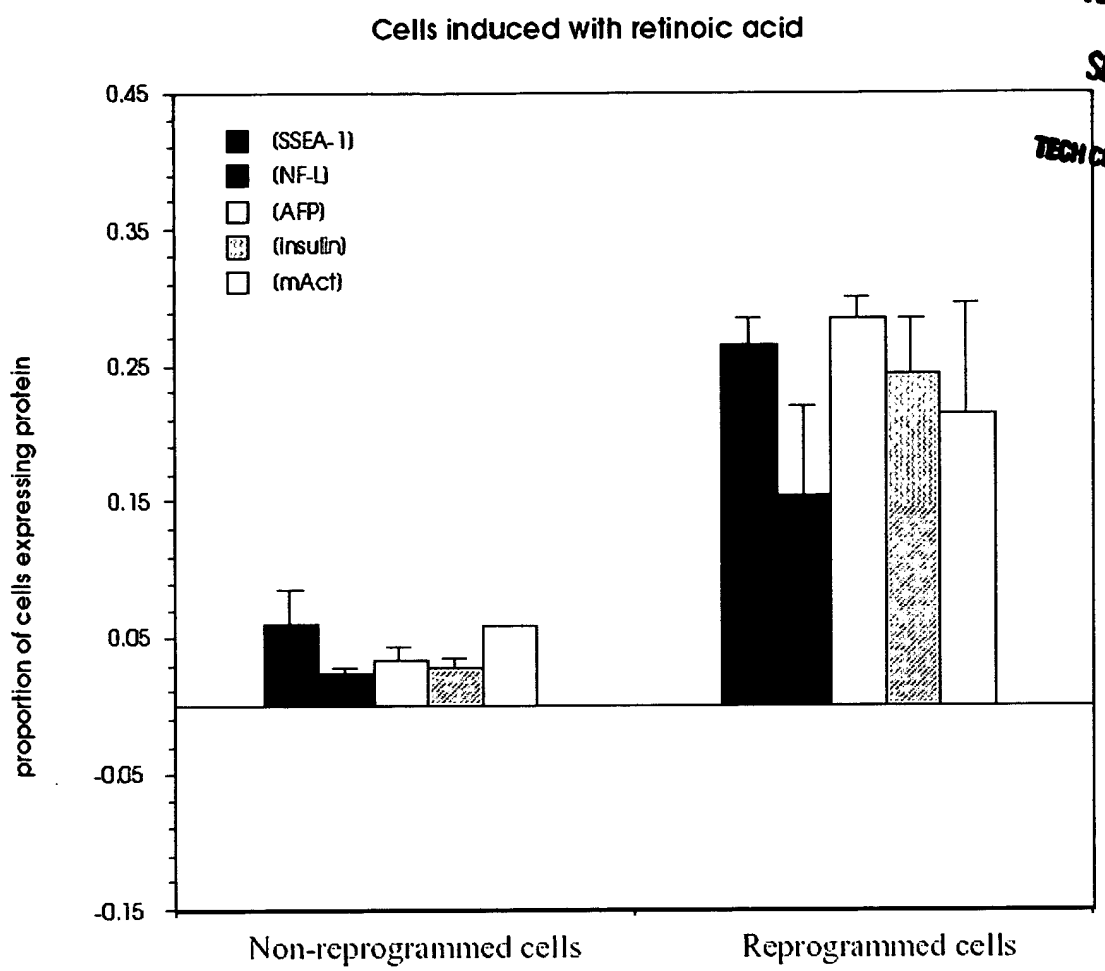

Figure 1: Immunostained Cells

Non-reprogrammed human keratinocytes ("Non-reprogrammed cells")and keratinocytes that were reprogrammed via treatment with 5-aza-2' deoxycytidine, trichostatin A and Tat-cyclin B ("Reprogrammed cells) were treated with 1 uM retinoic acid for 5 days. Cells were then fixed in 4% formaldehyde in phosphate buffered saline for 10 minutes, followed by 0.5% Triton-X100 for 30 seconds. Cells were washed in PBS then re-suspended in dilute horse serum (VectaStain® Kit, Vector Labs). Primary antibodies were added to a final concentration of 1:1000 and incubated for either 1 hour at 37°C or overnight at 4°C (mouse anti-neurofilament [neuron-specific; NF-L] , anti-á-fetoprotein [liver-specific; AFP], anti-insulin [beta cell-specific] and anti-cardiac actin [muscle-specific; mAct] were obtained from Santa Cruz Biotechnology; anti-stage specific embryonic antigen-1 [embryo-specific; SSEA-1] was obtained from the University of Iowa Hybridoma Center). Antibody detection was obtained using the VectaStain Kit (Vector Labs) following manufacturer's instructions. To determine the proportion of cells that express a protein, the percentage of stained cells in the appropriate "no primary" control was subtracted from the percentage of stained cells for each antibody. Each bar represents at least two experiments, n >300.

Reprogramed keratinocytes express the embryonic stem cell-specific Oct 4 mRNA

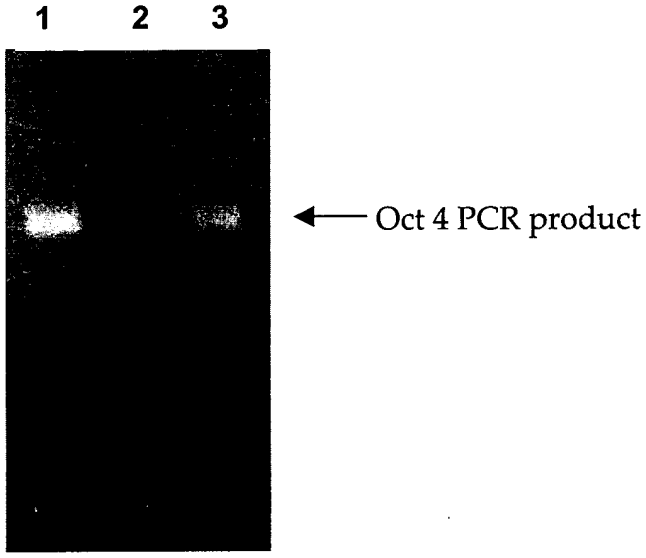

Figure 2: Ethidium bromide stained 2% agarose gel electrophoretogram. RT-PCR was performed on RNA extracted from human keratinocytes treated with 5-aza-2' deoxycytidine, trichostatin A and tat-cyclin B. RNA was extracted using TriZol®, following manufacturer's instructions. Total RNA was subjected to reverse transcription ("RT") using MMLV and human Oct4-specific RT primer (tccacccacttctgcag). 40 cycles of PCR were performed at an annealing temperature of T*m*–5°, wherein T*m* is of theOct4-specific amplification primers (caagggccgcagcttacacatgttc + cgrgaagctggagaaggagaagctg). *Lane 1:* PCR of remodeled keratinocytes with RT; *lane 2:* PCR of remodeled cells, no RT performed; *lane 3:* PCR of Oct4 cDNA obtained from stem cell cDNA library through the IMAGE Consortium.